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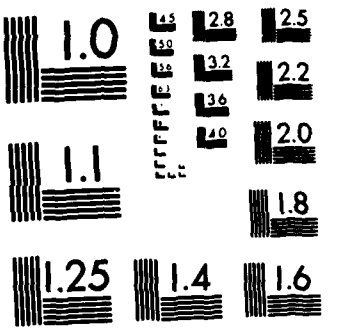

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Neohemoglobins and Cross-Linked Hemoglobins as Blood Substitute

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We have investigated the effect of heme modifications and specific cross-linking of β subunits in human and bovine hemoglobins. It appears that both techniques can be utilized for obtaining products potentially useful as oxygen carriers in resuscitative fluids.

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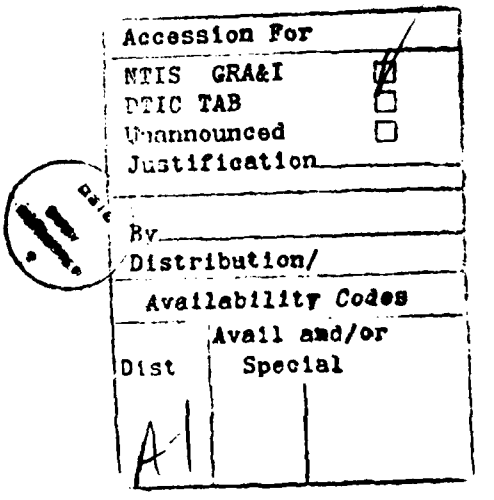

SUMMARY

Starting from deuteroporphyrin we synthetized 2,4-dibromo,2(or 4)-monocyano and 2-cyano-4-vinyl heme. These hemes were reinserted into apohemoglobin and the oxygen binding properties of the resulting hemoglobins were tested at various pH values between 6 and 8 in 0.05 M Tris or Bis-Tris buffer at 25° C. The oxygen affinity of 2,4-dibromo- and 2-cyano-4-vinyl hemoglobins was very similar to that of normal SFH. The oxygen binding cooperativity gave a value of n in the Hill plots between 1.8-2.0. The other hemoglobins had a somewhat higher oxygen affinity and a lower cooperativity with values of n near 1.5.

2,4-Dicyano-heme was also synthetized, however it failed to recombine with apohemoglobin.

Fumaryl, succinyl, and muconyl diaspirins were synthetized and utilized to produce intramolecular cross-linking of the subunits of human and bovine hemoglobins. After 2 hours at 37° C in 0.1 M bis-Tris buffer at pH 7.2, in the presence of 1 mM quantities of the various diaspirins, human hemoglobin reacted very well (70-90%) with the three reagents. Bovine hemoglobin showed a substantial reaction only with fumaryl diaspirin. The oxy, carbonmonoxy and deoxy derivatives of the two hemoglobins were used for the chemical treatment. The extent of cross-linking was tested by SDS gel electrophoresis. This analysis indicated that the major portion of the reacted hemoglobin was cross-linked producing subunits pairs, non dissociable upon denaturation in the presence of detergents.

The oxygen binding properties of these compounds were tested in the same buffers as above described.

For human hemoglobin, cross-linking of the deoxy derivative did not change the oxygen affinity and lowered only a little the binding cooperativity (values of n near 2.0). Instead, cross-linking of the deoxy derivative produced a compound with an oxygen

affinity lower than that of normal SFH.

For bovine hemoglobin, cross-linking of the oxy and carboxy derivatives increased substantially the oxygen affinity and eliminated the oxygen binding cooperrativity. Cross linking of the deoxy derivative left practically unchanged the oxygen affinity and only lowered a little the oxygen binding cooperativity of bovine hemoglobin (values of n near 2.0).

TABLE OF CONTENT

REPORT

MATERIALS AND METHODS

Stroma free-human and bovine hemoglobins were prepared by the filtration method. The respective heme-free proteins (apohemoglobins) were prepared by extraction with methyl-ethylketone (Teale, 1959). Chemical modification of deuteroporphyrins were obtained as described by Caughey et. al. (1966), Falk (1964), and as reported in the monography of Smith (1975). Some derivatives: 2,4-dibromo, 2-formyl-4-vinyl-, and 2 (or 4)-monocyano-deuteroporphyrin-IX-dimethyl ester were obtained from Mid-Century. All of the product prepared and purchased were chromatographically pure. Iron was inserted in the various porphyrins using a large excess of ferrous sulfate on the dimethyl ester derivatives. Recombination of apohemoglobin and neohemes was performed as described by Rossi-Fanelli et. al. (1958).

Acyl derivatives of 3,5-dibromosalicylic acid (diaspirins) were obtained as described by Zaugg et. al., (1980). Cross-linking by diaspirins was performed using a 2 molar excess of the reagent over tetrameric hemoglobin in 0.1 M tris buffer at pH between 7.2 - 7.4, for 2 hours at 37^{o}C. Oxy, carbonmonoxy and deoxy hemoglobins were used. Deoxygenation was obtained adding 1 mg/ml of Na^{+} dithionite to solutions previously flushed with nitrogen, in anaerobic environment. The extent of the reaction was followed by electrophoresis with the Beckman microzone system. Acylated hemoglobins had a faster anodic mobility. Polyacrylamide SDS gel electrophoresis (Walder et al, 1979) was used for monitoring the amount of cross-linked protein.

Recombined neohemoglogins and cross-linked hemoglobins were purified by chromatography on CM cellulose using a linear gradient formed by equal volumes of 0.01 M phosphate buffer at pH 6.2 and 0.04 M dibasic phosphate.

Densitometric scans of polyacrylamide gels and of microzone strips were performed with a Joyce and Loeble microdensitometer. Sedimentation velocities were measured in a Beckman model E analytical ultracentrifuge in 0.15 M tris buffer at pH 7.4, at temperatures between 18 and 20^{o}C. Oxygen equilibria were measured using a Gill's cell apparatus (Dolman and Gill, 1978) so to scan the absorption spectrum of the solutions at each step of oxygenation. Ferric hemoglobin formation was less than 5% of the total protein. The hemoglobin solutions were at concentrations between 5 and 10%. Temperature was controlled with a Digitek 500 digital thermometer.

RESULTS

Neohemoglobins

Fig. 1 shows the structure of heme where positions 2 and 4 are occupied by vinyl residues. These positions were variously substituted with electron withdrawing groups so to reduce the affinity of the iron in the heme for oxygen.

Neohemes used were 2,4-dibromo-, 2 (or 4)-mono-cyano-(the mixture of the 2 and 4 substituted hemes had not been resolved), and 2-cyano-4-vinyl-heme. In the mono-cyano-derivative the respective 4 and 2 positions were occupied by a proton.

Figs. 2 and 3 show the oxygen affinity of the neohemoglobins as compared to that on normal human SFH either in 0.05 M phosphate, or in 0.05 M Tris or in 0.05 M Bis-tris buffers at various pH values, at 25° C, in the absence of CO_2. It appears that in human hemoglobin the various neohemes tested were able at best to reproduce the oxygen affinity of untreated hemoglobin, with a lower cooperativity.

Figs. 2 and 3 also shows that there was specie specificity in the interaction of apohemoglobins with neohemes. In fact 2,4-dibromo-heme drastically increased the oxygen affinity of bovine hemoglobin, which normally is even lower than that of human SHF. Also it abolishes the heme-heme interaction. In horse hemoglobin 2,4-dibromo-heme appears to produce a hemoglobin with a lower oxygen affinity than human SHF. Untreated horse and human hemoglobins have identical oxygen affinities.

Cross-Linked Human and Bovine Hemoglobins

For this reaction we have used the bio-3,5-dibromo-salicylic derivatives of fumaryl, succinyl and muconyl residues, whose structural formulas are illustrated in fig 4.

The extent of reaction, estimated from microzone electrophoresis, was very high for human hemoglobin, reaching the 80-90% of the total for both the liganded and unliganded derivatives. Bovine hemoglobin was less reactive. The fumaryl-diaspirin produced a reaction of 60-70%. The succinyl and muconyl diaspirins produced only 30% reaction or less.

The extent of cross-linking estimated by SDS gel electrophoresis indicated that in non-purified samples the extent of cross-linking was less, about 2/3, than that expected from the extent of the reaction with the diaspirins. After purification by chromatography this discrepance was corrected and the amount of cross-linked protein approached the 50% mark expected from cross-linking of the subunits only as described by Walder et. al. (1980).

Native and cross-linked samples of human and bovine hemoglobins were indistinguishable in sedimentation velocity experiments which in any case produced single symmetrical peaks with a sedimentation constant near $S_{20,w}$= 4.2, independent of protein concentration.

Functional tests of cross-linked hemoglobins are presented in Fig. 5. Circles refer to human and triangles to bovine hemoglobin. Closed symbols imply cross-linking of the oxy-derivatives. For human hemoglobin, hemoglobin cross-linking of the oxy derivative left the oxygen affinity practically unchanged, while cross-linking of the deoxy

derivative produced a substantial decrease of the oxygen affinity. The value of n in the Hill plots was near 2.7 for native hemoglobin and between 1.6 and 1.9 for cross-linked non purified samples. For bovine hemo globin cross-linking of the oxy derivative produced a substantial increase of the oxygen affinity, while in the deoxy derivative it left the oxygen affinity practically unchanged. The values of n in the Hill plots were near 2.8 for the native protein and between 1.5 and 1.9 for unpurified samples.

Purification by chromatography of human or bovine hemoglobins cross-linked as deoxy derivative, seemed to increase the value of n in the functional tests.

CONCLUSIONS

The presence of the porphyrin ring of the heme of electron withdrawing groups is expected to lower the affinity of the iron atom for ligands. The neohemes here investigated show that at least in human hemoglobin the mere substitution of the vinyl groups in position 2 and 4 with other electron withdrawing agents is not enough for reducing the oxygen affinity of the protein to levels desirable in blood substitutes.

In regard to cross-linking with diasprinis, the decreased oxygen affinity of human hemoglobin, when reacted as unliganded derivative, is extremely interesting and promising.

The drastic increase of the oxygen affinity of bovine hemoglobin upon cross-linking of its liganded form, implies a distortion of the molecule, which is not produced when the reaction is performed on the deoxy derivative. In this case the oxygen binding cooperativity is decreased, however it is still present and the oxygen affinity remains lower than tha tof human SHF, making the protein suitable as blood substitute. The tetrameric structure of the proteins stabilized by cross-linking should allow a longer retention time after transfusion than for untreated human SHF.

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LEGENDS

Fig. 1 Ferroprotoporphyrin-IX

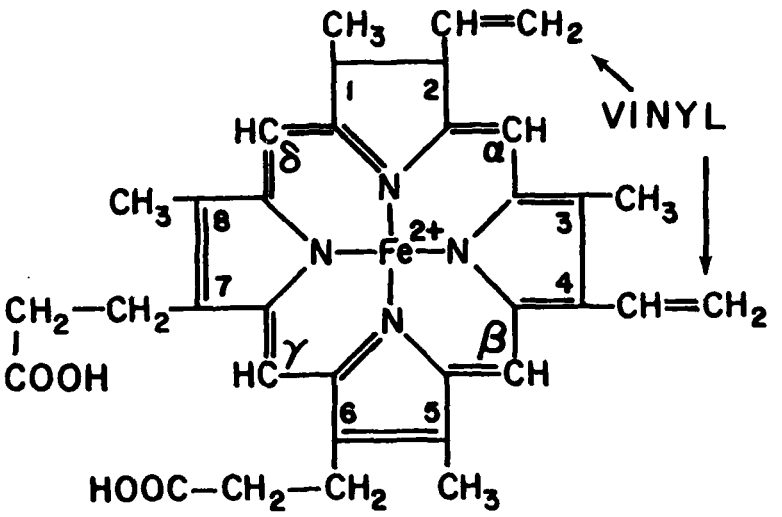
Fig. 2 Oxygen binding characteristics of the indicated reconstituted hemoglobin in 0.05 M Tris or Bis-Tris buffers at 25°C in the prssence of 5% CO_2. The symbol A refers to human SFH.

Fig. 3 Oxygen binding characteristics of the indicated reconstituted hemoglobins in 0.05 M phosphate buffers at 25° C, in the presence of 5% CO_2. The symbol A refers to human SFH.

Fig. 4 Various esters of bis-3,5-dibromosalicylic acid.

Fig. 5 Oxygen binding characteristics of various cross-linked hemoglobins. A refers to normal human SFH and B to normal bovine SFH. Δ, ▲)Bovine hemoglobin, O,●)human hemoglobins. Open symbols refer to cross-linking reactions performed on the deoxyderivatives of human or bovine hemoglobin.

Fig. 1



Ferroprotoporphyrin-IX (heme)

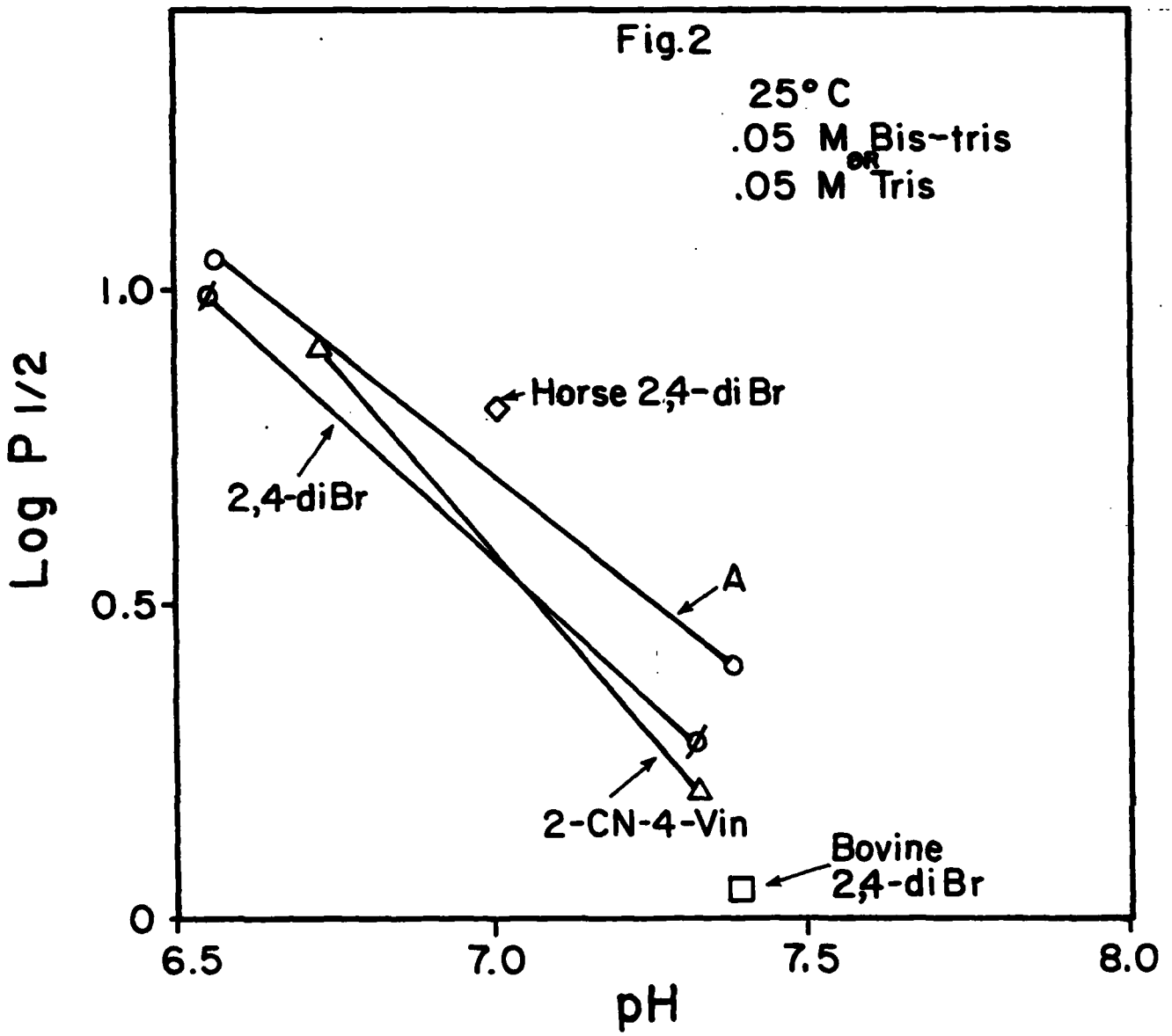


Fig.2
25° C
.05 M Bis-tris
or
.05 M Tris
Log P 1/2
1.0
0.5
0
Horse 2,4-di Br
2,4-diBr
A
2-CN-4-Vin
Bovine
2,4-di Br
6.5
7.0
7.5
8.0
pH

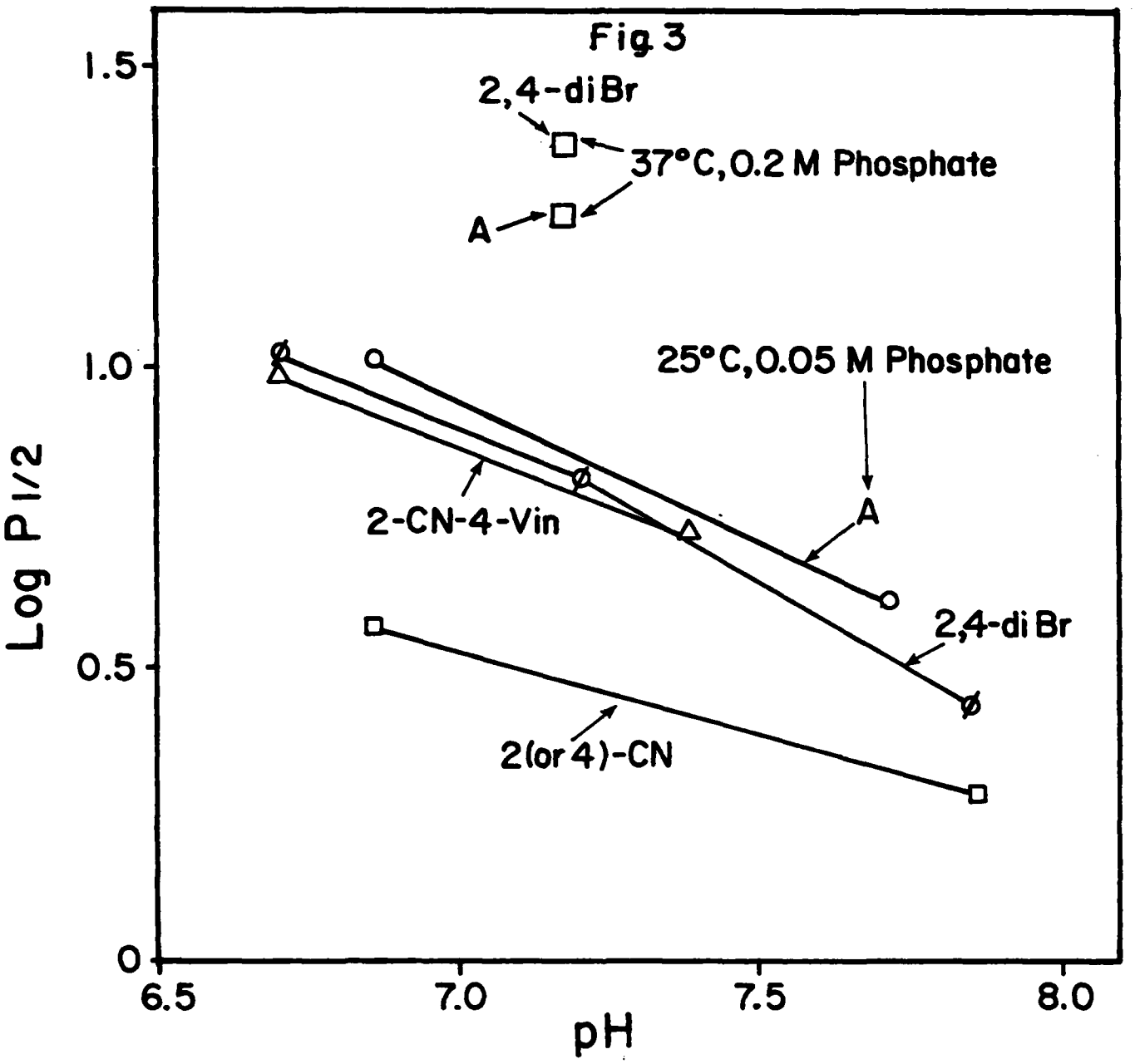
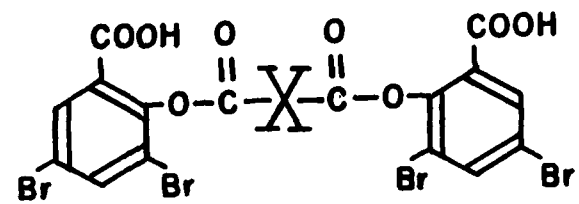


Fig 3
2,4-diBr
37°C, 0.2 M Phosphate
A
25°C, 0.05 M Phosphate
A
2-CN-4-Vin
2,4-di Br
2(or 4)-CN
Log P1/2
pH
1.5
1.0
0.5
0
6.5
7.0
7.5
8.0

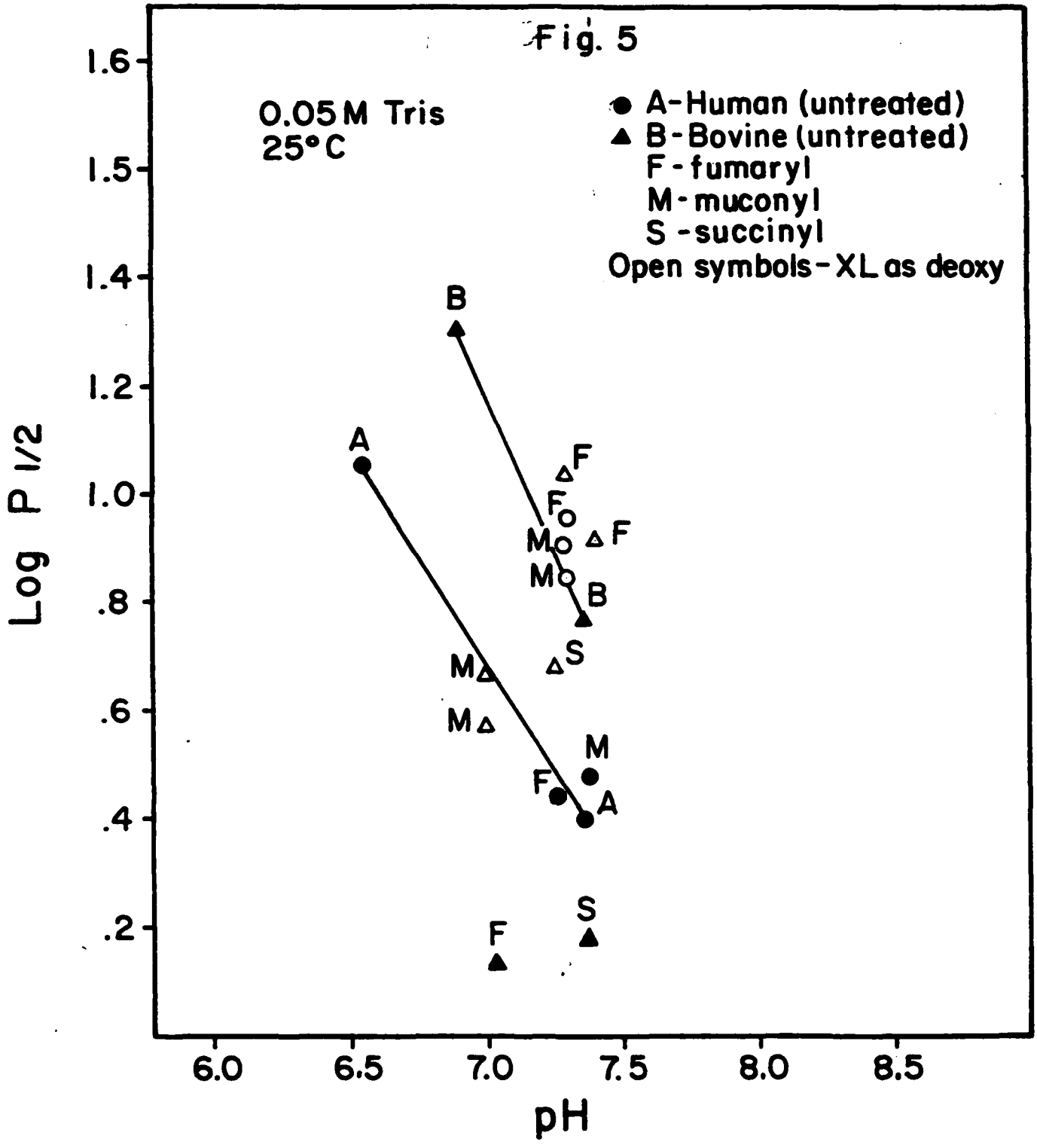
Fig. 4



BIS-3,5-DIBROMOSALICYLIC ACID

X = CH_2-CH_2 SUCCINYL
CH=CH FUMARYL
CH=CH-CH=CH MUCONYL

Fig. 5


0.05 M Tris
25°C
● A-Human (untreated)
▲ B-Bovine (untreated)
F - fumaryl
M - muconyl
S - succinyl
Open symbols - XL as deoxy
Log P 1/2
1.6
1.5
1.4
1.2
1.0
.8
.6
.4
.2
6.0
6.5
7.0
7.5
8.0
8.5
pH

END

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